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PRINCIPAL INVESTIGATOR: Beate Lanske

CONTRACTING ORGANIZATION: Harvard University
Boston, MA 02115-6027

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Beate Lanske

E-Mail: beate_lanske@HSDM.Harvard.edu

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President & Fellows of Harvard College
Sponsored Programs Administration, 25 Shattuck St., Boston Ma 02115

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**14. ABSTRACT**

The most significant findings from the last period are the following.

1) We are the first to show that Klotho is expressed in proximal tubules and that *in vivo* deletion of Klotho from proximal tubules leads to reduced urinary phosphate wasting (AIM 2). Three mouse models were established to confirm our findings. It seems, however, that there is an interrelationship between proximal and distal tubules since deletion of Klotho from either target tissue leads only to a mild phenotype, so compensatory mechanisms are suggested.
2) In AIM3 we found that Klotho expression in bone forming cells is required to induce FGF23 transcription upon renal failure. Such findings can ultimately lead to therapeutic interventions to block the increase in FGF23 production during renal failure. Here two mouse models resulted in similar results, so we are very confident about our results.

**15. SUBJECT TERMS**
**Nothing listed**

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# 1. Introduction

We are conducting a study regarding the underlying genetic and endocrine mechanisms for Osteoporosis and Related Bone Disease such as skeletal mineralization defects related to chronic kidney failure. Osteoporosis is a significant health problem. It is estimated that 44 million Americans, nearly 70% of which are women, are affected by this disease. It is of particular concern to the Veteran's Administration since women are now the fastest growing segment of veterans. Our work and the work of others have revealed much about the complex physiology of bone development and maintenance including the endocrine regulation of mineral homeostasis that is absolutely necessary for skeletal health. Hormonal factors from bone, kidneys and parathyroid glands form an interactive network to maintain a tight balance of minerals in the body. Our proposal is to develop a better understanding of how one protein in particular, Klotho, specifically contributes to bone mineralization defects. We hypothesize that tissue-specific reduced expression of Klotho is responsible for the bone mineralization defects present in patients with osteoporosis and/or chronic kidney disease. To investigate this hypothesis we have experimentally deleted Klotho expression in parathyroid glands, kidneys, and bone independently to determine 1) how each organ contributes to the disease processes and 2) the relative contribution of each organ to the mineralization defects. We are using our transgenic mouse that utilizes Cre-lox technology to selectively ablate Klotho in each organ and analyze the effects to support our hypothesis.

## 2. Keywords

Klotho, FGF23, PTH, CKD-MBD, Osteoporosis, Mineral Homeostasis, osteopontin, adenine, resorption, FGFR1, ablation, renal failure, parathyroid gland, kidney, bone, phosphate, calcium, uremia.

# 3. Accomplishments

## Major Goals and Tasks

To test our hypothesis and *attain* our overall aim of delineating the tissue-specific role of Klotho in osteoporosis, CKD-MBD and other bone pathology, we proposed the following specific aims. Each aim is designed to study a specific tissue using floxed Klotho (*Klotho*$^{fl/fl}$) mice crossed with a mouse line carrying the Cre recombinase gene driven by a tissue specific promoter. Mice from these crosses have Klotho ablated from the target tissue by Cre-LoxP recombination.

SA1: Parathyroid gland-specific deletion of Klotho: Klotho has been reported to mediate the suppression of PTH by FGF23. In addition, Klotho has been shown to recruit the Na+,K+-ATPase to the cell surface upon changes in calcium concentrations in the parathyroid gland. It has been suggested that a decrease in Klotho expression in the parathyroid gland is responsible for the induction of secondary hyperparathyroidism due to a resistance to FGF23 suppression of PTH transcription/secretion in CKD-MBD. Specific deletion of *Klotho* from the parathyroid gland is therefore an important prerequisite to obtain novel information about Klotho's organ-specific function in mineral ion homeostasis. *Klotho*$^{fl/fl}$ mice will be crossed with *PTHcre* mice to generate mice with Klotho specifically ablated in the parathyroid glands. We will compare *PTHcre;Klotho*$^{fl/fl}$ and their control *Klotho*$^{fl/fl}$ littermates under normal conditions and then under disease conditions by feeding them an Adenine diet or optionally by 5/6 nephrectomy to induce CKD. The results obtained from this study will provide evidence whether Klotho signaling in the parathyroid glands has a critical role in bone mineral defects.

SA2: Kidney-specific deletion of Klotho: Renal expression of Klotho is important for normal mineral ion homeostasis. To examine the consequence of loss of renal Klotho function from the entire kidney, *Klotho*$^{fl/fl}$ mice will be crossed with *Six2cre* transgenic mice; Jax #009606-Six2-EGFP/cre1Amc/J. Six2-expressing cap mesenchyme represents a multipotent nephron progenitor population; therefore deletion of Klotho is anticipated throughout the kidney. Moreover, we plan to refine our data to determine whether Klotho expression in proximal or distal tubules is more critical for CKD-MBD. We have recently demonstrated that Klotho is expressed not only in distal tubules but also in proximal tubules of the kidney. This is an important finding because prior to these data it was unclear how Fgf23 could downregulate the sodium phosphate co-transporters (NaPi2a, NaPi2c) and inhibit expression of the 1α(OH)ase in proximal tubules if Klotho was expressed only in distal tubules. We will investigate the importance of Klotho functions in both tubules by separately ablating Klotho from each cell type. In order to identify Klotho's potential role in proximal tubules to regulate phosphate and vitamin D homeostasis, *Klotho*$^{fl/fl}$ mice will be crossed with kidney androgen-regulated protein (*KAP)cre* transgenic mice. To investigate Klotho's function in distal tubules where it has been reported to induce renal calcium re-absorption by its actions on TRPV5 *Klotho*$^{fl/fl}$ mice were crossed with mouse cadherin 16 (*Ksp 1.3 kb);cre* mice (article published by JASN; see preliminary data). Induction of CKD in this mouse strain will determine whether renal Klotho is involved in the progression of bone mineralization defects and to what extent this involvement derives from the proximal versus distal tubules. We will compare kidney-specific *cre;Klotho*$^{fl/fl}$ and their control *Klotho*$^{fl/fl}$ littermates under normal conditions and then under disease conditions by feeding them an Adenine diet or optionally by 5/6 nephrectomy to induce CKD. The results obtained from this study will provide evidence whether Klotho signaling in the kidney has a critical role.

SA3: Bone-specific deletion of Klotho: We and others have recently found that Klotho is expressed at low levels in osteoblasts and that *Klotho*$^{-/-}$ mice exhibit a severe mineralization defect *in vivo* and *in vitro*. Most importantly, we have recently discovered that Klotho and PTH function independently of FGF23 to affect bone mineralization. To determine the mechanism that underlies this previously unknown function of Klotho in bone mineralization, we will cross *Klotho*$^{fl/fl}$ mice with various cre transgenic mice (*Prx1cre, Col 1(α1)cre, and DMP1cre)* to generate mice with Klotho specifically ablated in the bone. The result obtained from this study will elucidate whether loss of Klotho expression from bone contributes to the mineralization defect observed in osteoporosis, CKD-MBD and other diseases. We will compare bone-specific *cre;Klotho*$^{fl/fl}$ and their control *Klotho*$^{fl/fl}$ littermates under normal conditions and then under disease conditions by feeding them an Adenine

diet or optionally by 5/6 nephrectomy to induce CKD. The results obtained from this study will provide evidence whether Klotho signaling in the bone has a critical role.

The following set of tasks will be performed for each of the transgenic mouse models listed. Note: the tasks are not completely linear and a subsequent task may begin prior to the end of the previous task.

Task 1: Generate/Maintain Mice (6 months)

- Cross *Klotho*$^{fl/fl}$ with appropriate cre transgenic mice to generate required genotypes.
  Aim 1: **PTHcre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
  Aim 2: *Six2cre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
  *KAPcre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
  **KSPcre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
  Aim 3: *Prx1cre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
  *Col 1(α1)cre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
  *DMP1cre;Klotho*$^{fl/fl}$ *and Klotho*$^{fl/fl}$ control littermates
- Confirm ablation of Klotho using immunohistochemistry and qPCR
- Maintain breeding pairs/Breed experimental mice as needed
  6 mice, both males and females, at birth-P2, 3, 6-9 weeks, and 22 weeks postnatally

  *NOTE: The *PTHcre;Klotho*$^{fl/fl}$ and *KspCre;Klotho*$^{fl/fl}$ mouse lines were generated and characterized prior to the start of the project, but had not been challenged with induction of CKD.

Task 2: Analyze/Compare Mutant Mice to Control Littermates Under Normal Conditions at All Ages (18-21 months)

- *Macroscopic analysis:* morphology, weight, size, survival, health status
- *Biochemical analysis:* serum and urinary parameters
- *Routine Histology:* bone and soft tissues; mineralization (normal and ectopic), proliferation, apoptosis
- *Quantitative analyses:* histomorphometry (bone formation rate/BFR), μCT, or pQCT
- *Gene expression studies:* Nanostring or qRT-PCR in bone, kidney and parathyroid glands
- *Protein expression studies:* Western Blot and Immunohistochemistry in bone, kidney and parathyroids
- *In vitro* analysis: Mineralization using Bone Marrow Stromal Cells and Calvarial Cells, Isolation of Distal Tubular Segments for in vitro analyses
- Statistical Analyses of all Data

Task 3: Challenge Mice with Induction of CKD by either feeding Adenine Diet or 5/6 nephrectomy (7-9 months)

- Produce sufficient litters and genotype all pups
- *Adenine Diet:* Mutant Mice and Klotho$^{fl/fl}$ control mice (see Task 1) will be fed a control diet *(DYETS #I10457 - Modified AIN-76A Purified Rodent Diet with 0.9% Phosphorus and 0.6% Calcium)* with or without addition of adenine for a period of 4 weeks. The mice will start on the diet at weaning (3 weeks old).
- *5/6 nephrectomy:* This procedure will only be performed if the Adenine Diet is not sufficient to induce CKD. We do not expect this since we have already confirmed that the protocol we have established for the Adenine Diet is effective. Nevertheless, as backup plan we would remove 2/3 of the first kidney at between 9-12 weeks of age. The second kidney would be completely removed one week later.
- All Mice will be monitored for adverse side-effects.

Task 4: Analyze Mice Under Disease Conditions (18-21 months)
Subtasks are the same as Task 2 above

**Accomplishments**

The results of our work for the previous period are summarized here, organized by specific mouse line. Specific target dates were not given in the SOW (see above). We report the percentage of work completed per mouse line based on the task times listed above.

**Aim 1 - *PTHCre; Klotho$^{fl/fl}$* Mice**

This Aim was completed and the work was finalized and published in December 2013 together with our collaborators.

*(Olauson H, Lindberg K, Amin R, Sato T, Jia T, Goetz R, Mohammadi M, Andersson G, **Lanske B**, Larsson TE. Parathyroid-specific deletion of Klotho unravels a novel calcineurin-dependent FGF23 signaling pathway that regulates PTH secretion. PLoS Genet. 2013 Dec;9(12):e1003975).*

**Aim 2 - *KSPcre;Klotho$^{fl/fl}$* Mice**

As noted in the progress report last year, we have dropped this line of mice. This is due to a publication on this topic by one of our former collaborators.

*(Olauson H, Lindberg K, Amin R, Jia T, Wernerson A, Andersson G, Larsson TE. Targeted deletion of Klotho in kidney distal tubule disrupts mineral metabolism. J Am Soc Nephrol. 2012 Oct;23(10):1641-51).*

**Aim 2 – *Six2cre;Klotho$^{fl/fl}$* Mice**

This project is now considered complete and was published in *JASN*.

*(Lindberg K, Amin R, Moe OW, Hu MC, Erben RG, Ostman Wernerson A, **Lanske B**, Olauson H, Larsson TE. The Kidney Is the Principal Organ Mediating Klotho Effects. J Am Soc Nephrol. 2014 Oct;25(10):2169-75).*

**Aim 2 - *KAPcre;Klotho$^{fl/fl}$* Mice**

We first re-confirmed our previous observation about the renal expression pattern of Klotho using immunohistochemistry. Klotho staining was detected in the renal cortex, but not in the medulla, of wild-type mice (Figure 1A, left panel). In the cortex, distal tubules had a strong positive staining whereas in proximal tubules the staining was substantially weaker (Figure 1A, right panel). We then confirmed the expression of Klotho mRNA using RNAScope, a novel method for *in situ* hybridization. Klotho RNA was predominately expressed in the distal tubules, with weaker but positive expression in proximal tubules (Figure 1B), consistent with the immunohistochemistry results.

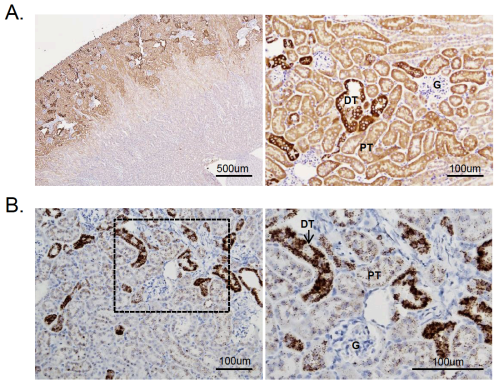



Figure 1: (A) klotho protein expression and (B) Klotho mRNA expression. PT = proximal tubules, DT = distale tubules, G = glomerulae.

**Characterization of Kap-KL mice**

We investigated the importance of Klotho in proximal tubules by generating mice expressing Cre recombinase under the Kap promoter. AS stated in the last progress report, this was the best characterized proximal tubular-specific Cre strain available through public mice repositories. Cre expression in these mice has previously been shown to be restricted to the proximal tubules. To analyze the recombination specificity and efficiency of this line, mice were crossed with a tdTomato reporter strain. For our experiments we used *KapCre:Klotho$^{fl/fl}$;td Tomato$^{fl/+}$ (Kap-KL)* and *KapCre;tdTomato$^{fl/+}$ (Kap-WT)* mice. *Kap-KL* mice had a normal gross phenotype (Figure 2A) and survived as well as wild type littermates. First, we confirmed the deletion of Klotho from proximal tubules using immunohistochemistry (Figure 2B). We also evaluated isolated kidney proximal tubular cell homogenates and observed that Klotho mRNA expression was reduced by 66% (p=0.02, n=6) (Figure 2C) and Klotho protein expression was reduced by 52% (p=0.02, n=6) (Figure 2D). Next, we evaluated the expression of key renal genes by quantitative PCR (qPCR). Transcript levels of Npt2a were significantly increased in *Kap-KL* mice, whereas Npt2c expression levels were unchanged. Both Cyp27b1 and Cyp24a1 were significantly increased in *Kap-KL* mice (n=4-6)(Figure 2E). Western blot analysis revealed significantly

increased Npt2a expression at the brush border membrane (BBM) in *Kap-KL* mice compared with *Kap-WT* mice (p=0.003, n=3) (Figure 2F).

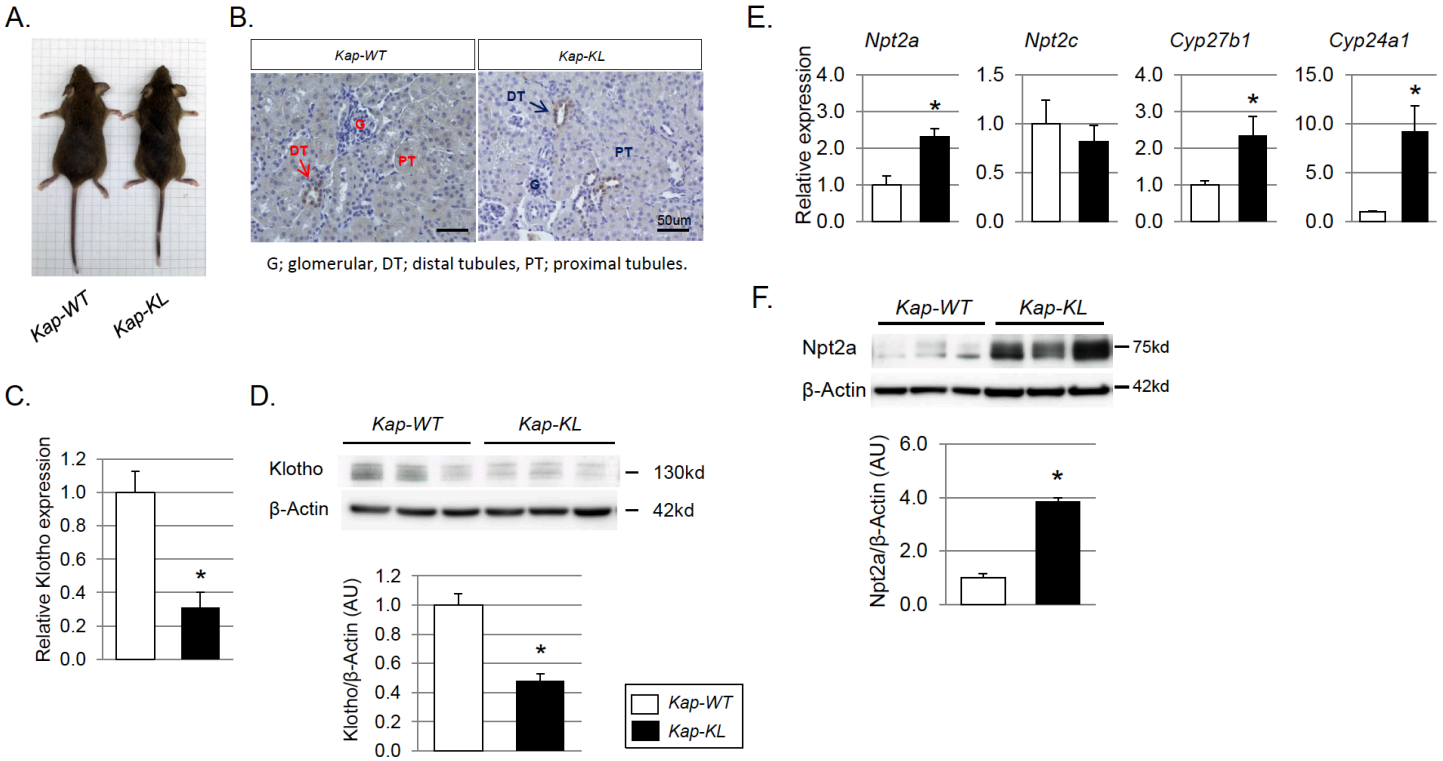



Figure 2: Characterization of Kap-KL mice. (A) *Kap-KL* (left) mice and *Kap-WT* (right) mice had a normal gross appearance. (B) Immunohistochemical staining of Klotho revealed partial deletion of Klotho in *KL-KAP* mice kidney. (scale bar; 50µm). Klotho mRNA and protein levels were measured by qPCR (C) and western blot analysis (D) from isolated kidney proximal tubules of *Kap-KL* and *Kap-WT* mice. (n=6). (E) Relative mRNA levels of renal transcripts *(Npt2a, Npt2c, Cyp27b1*, and *Cyp24a1)* were measured by qPCR in *Kap-KL* mice and *Kap-WT* mice. (n=4-6). (F) Npt2a expression was detected by Western blot analysis. Brush border membrane vesicles (BBMVs) were isolated from the kidney of *Kap-KL* and *Kap-WT* mice. β-Actin was used as an internal control. (*p=0.003*, n=3). Data represent the means ± SEM.

**Kap-KL mice on high phosphate challenge**

We originally planned to challenge this mouse strain with induced CKD in order to determine whether renal Klotho is involved in the progression of bone mineralization defects and to what extent this involvement derives from the proximal versus distal tubules. However as demonstrated in the last progress report, under normal conditions, there was no significant change in urine and serum levels of calcium and phosphate, nor in their regulatory factors (FGF23 and 1,25(OH)$_2$D), except that serum PTH concentration significantly increases in *KAP-KL* mice. It was decided that both the adenine-induced CKD model and 5/6 Nephrectomy model were too severe to detect the milder role of Klotho in proximal tubules. Therefore, we focused on phosphate handling, which is assumed to be a key role of Klotho in proximal tubules and put further stress on renal phosphate handling. Both *Kap-KL* and *Kap-WT* mice were challenged with high phosphate (HP) in the drinking water for 8 weeks. Serum calcium and phosphate levels remained unchanged in both *Kap-KL* and *Kap-WT* (n=3-5) (Figure 3A). Importantly, *Kap-WT* mice substantially increased their urinary phosphate excretion after HP loading, however urinary phosphate excretion in *Kap-KL* mice on HP remained unchanged (n=5) (Figure 3B). This effect was not due to decreased renal function as serum creatinine and BUN were similar between groups (ns, n=3-5) (Figure 3C). Serum Fgf23 were significantly increased in both *Kap-KL* and *Kap-WT* mice on HP whereas 1,25(OH)$_2$D levels were significantly increased only in *Kap-KL* on HP. PTH levels increased significantly in *Kap-WT* mice on HP when compared to unchallenged mice, whereas no changes were noted in *Kap-KL* mice (Figure 3D).

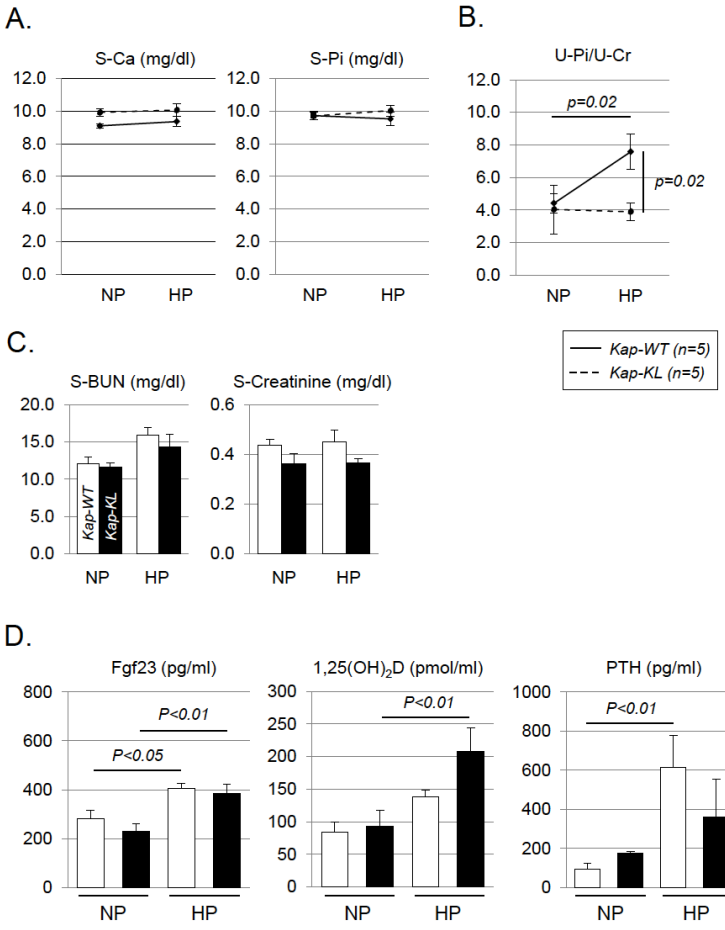



Figure 3: Serum and urine biochemistry was analyzed in *Kap-KL* and *Kap-WT* mice pre- and post- high phosphate drinking water (10mM phosphate for 8 weeks). (A) Analysis of serum calcium and phosphate, (B) and urinary phosphate/creatinine. (C) Serum BUN and creatinine. (n=3-6, ns). (D) Serum Fgf23, $1,25(OH)_2D$ and PTH.

Interestingly, proximal tubular damage was observed only in *Kap-KL* mice on HP. Such anomalies could not be observed in *Kap-WT* mice on HP, nor in the unchallenged condition in both *Kap-WT* mice and *Kap-KL* mice (n=4-7) (Figure 4).

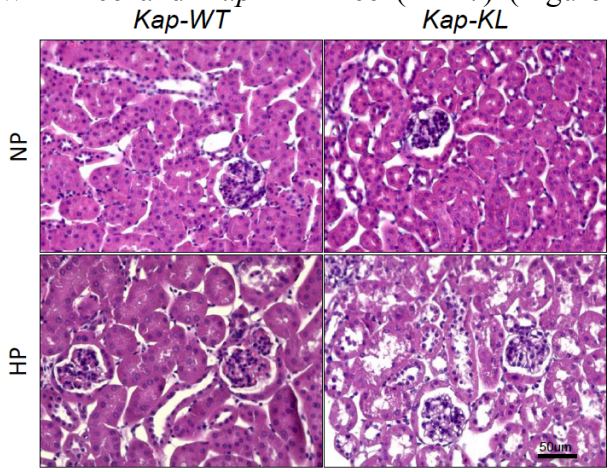



Figure 4: Histological paraffin sections of kidneys from *Kap-WT* and *Kap-KL* mice fed normal (NP) or high phosphate (HP) drinking water.

**Other proximal tubular-specific Cre strains**

We generated and tested additional mouse models to verify our observations in KAP Cre mice. Floxed Klotho mice (*Klotho*$^{fl/fl}$) were crossed with mice expressing Cre recombinase under the phosphoenolpyruvate carboxykinase (*PEPCK*$^{Cre}$), and the sodium phosphate cotransporter-2a1 (*Slc34a1*$^{Cre}$) promoter, respectively.

Table.1

| | KAP-KL | PEPCK-KL | SLC34a1-KL |
|---|---|---|---|
| **Serum** | | | |
| Calcium | – | – | ↑ |
| Phosphate | – | – | ↑ |
| Fgf23 | – | ↑↑ | – |
| PTH | ↑ | – | – |
| $1,25(OH)_2D$ | – | – | – |
| **Urine** | | | |
| Ca | – | – | – |
| Pi | – / ↓ * | ↓ | ↓↓ |
| **BBM** | | | |
| NaPi2a | ↑↑↑ | ↑↑ | ↑ |

Each strain is compared with its appropriate wild-type controls

– ; unchanged and/or normal

* ; on high phosphate drinking water

As shown in Table1, *PEPCK-KL* and *Slc34a1-KL* mice also showed decreased urinary phosphate excretion and an increase in Npt2a expression at the BBM. In terms of vitamin D homeostasis, serum $1,25(OH)_2D$ levels were unchanged under basal conditions in *PEPCK-KL and Slc34a1-KL mice*.

All three models displayed impaired urinary phosphate excretion and increased abundance of Npt2a in the brush border membrane. Notably, the knockout mice had no or only mild hyperphosphatemia, indicating the presence of compensatory mechanisms. Effects on $1,25(OH)_2D$ varied between strains but were overall modest, challenging the current view that intact Fgf23-Klotho signaling in the proximal tubules is crucial for vitamin D regulation.

We demonstrate that proximal tubular Klotho is important for renal phosphate reabsorption and that deletion of proximal tubular Klotho leads to hypophosphaturia. There is a definite, but limited, role for Klotho in renal proximal tubules. Deletion of Klotho from either distal or proximal tubules alone results in only minor changes in mineral homeostasis, suggesting an integrated and intricate cooperation between these two expression loci. The interplay between proximal and distal tubule is controlling mineral metabolism. Moreover, our data also suggest that Klotho expressed in the proximal tubules protects phosphate induced proximal tubular damage.
The data of this Aim were submitted to *Kidney International* for publication and the manuscript is currently under revision and will be resubmitted during the next year.

**Aim 3 – *Prx1cre;Klotho$^{fl/fl}$* Mice**

This aim is to investigate how bone-specific changes in Klotho expression affect mineral ion homeostasis and the progression of bone mineralization defects in chronic kidney disease. For this purpose, floxed Klotho mice were crossed with mice expressing Cre recombinase under the control of the Prx1 promoter to create a mouse in which *Klotho* is deleted in early limb mesenchyme.

We have continued to analyze the phenotype of control and mutant mice during the past year and are now ready to submit the data for publication. We have examined the phenotype of *Klotho$^{fl/fl}$* and *Prx1cre;Klotho$^{fl/fl}$* mice at 6 weeks of age and at 16 weeks of age after challenge with control and Adenine diet or sham and 5/6 NTX to induce renal failure. The data are shown below.

***6-week old mice -*** *In the previous report we noted that mice have lower BV/TV and connectivity.*
Histomorphometric examinations of male femurs stained with toluene blue showed no statistically significant differences in trabecular bone volume or connectivity between groups for the selected region of interest. However, there was a trend, similar to findings from μCT, that *Prx1cre;Klotho$^{fl/fl}$* mice have less trabecular bone volume and trabecular thickness in the distal femur than *Klotho$^{fl/fl}$* mice. Increased bone resorption may exacerbate the loss of trabecular bone volume and connectivity of *Prx1cre;Klotho$^{fl/fl}$* mice, so we assessed osteoclast number and activity by histomorphomtry, qRT-PCR and serum biochemistry analyses. TRAP staining of decalcified methylmethacrylate femur sections showed normal osteoclast number and surface in *Prx1cre;Klotho$^{fl/fl}$* mouse femora compared to those in *Klotho$^{fl/fl}$* mice. Gene expression analyses revealed that there are no significant differences in RANK mRNA expression between groups. However, biochemical measures of collagen breakdown revealed that *Prx1cre;Klotho$^{fl/fl}$* mice have markedly reduced amounts of type I collagen fragments (CTX) in circulation compared to *Klotho$^{fl/fl}$* mice. Since collagen breakdown is a measure of bone resorption these findings revealed that despite no changes in osteoclast number or activity *Prx1cre; Klotho$^{fl/fl}$* mice have reduced bone resorption. It is well documented that osteoblasts regulate osteoclastogenesis through the production of cytokines, including the pro-osteoclastogenic factor RANKL and the anti-osteoclastogenic decoy receptor OPG. We therefore measured the expression of RANKL and OPG in limbs of *Klotho$^{fl/fl}$* and *Prx1cre;Klotho$^{fl/fl}$* mice using qRT-PCR. The RANKL/OPG ratio is a key determinant of the extent of osteoclastogenesis. The ratio of RANKL to OPG expression in long bones of Prx1-Cre; Klotho$^{fl/fl}$ mice was reduced by <50%, suggesting that diminished osteoclast function results at least in part from aberrant osteoblast signaling.
Histomorphometric analyses of male femurs revealed that there were no significant differences in osteoblast number or surface between groups. However, the expression of several osteoblast transcripts including Runx2, Col1α1, Osx, OC and ALP were significantly increased in *Prx1cre; Klotho$^{fl/fl}$* femurs. Since type I collagen is the most abundant structural protein in bone, we then analyzed protein expression of type I collagen in femurs extracted from *Klotho $^{fl/fl}$* and *Prx1cre;Klotho$^{fl/fl}$* mice. Surprisingly, femurs of *Prx1cre;Klotho$^{fl/fl}$* mice had a significantly higher Col1α1 mRNA and protein (Figure 5) expression when compared to *Klotho$^{fl/fl}$* mice.

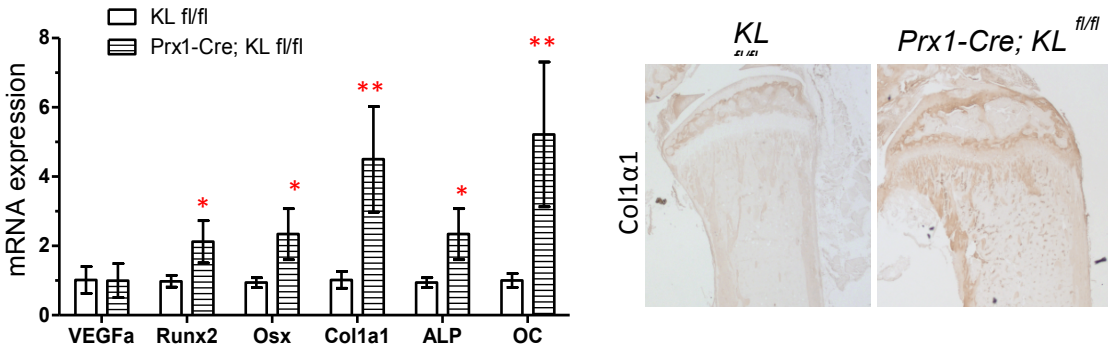



Figure 5: (left) mRNA expression of osteoblast markers by qRT-PCR. (right). Immunohistochemistry for Collagen Type α1 on femoral sections of *Klotho*$^{fl/fl}$ and *Prx1cre; Klotho*$^{fl/fl}$ mice.

Polarization microscopy of Sirius-red stained collagen further revealed that type I collagen fibrils in cortical bone of *Prx1cre;Klotho*$^{fl/fl}$ mice are randomly oriented and greatly contrast the birefringent, lamellar organization of *Klotho*$^{fl/fl}$ bone. This disorganization of type I collagen is associated with low bone mass – which we see in *Prx1cre;Klotho*$^{fl/fl}$ mice. We are currently trying to better understand the molecular pathways by which conditional deletion of Klotho affects collagen organization.

***Data from 16-week old mice challenged with adenine diet or 5/6 NTX to induce renal failure***

We found that kidneys of adenine fed *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice have strikingly higher expression of p16$^{INK4a}$ tumor suppressor than kidneys of control fed animals and significantly lower renal Klotho expression. These data are consistent with a recent paper published in Science that showed that p16$^{INK4a}$ is an upstream repressor of α Klotho (Sato et al. Nat Commun. 2015. 6:6035.).

There is accumulating evidence that bone mineralization defects in physiological aging and CKD are associated with reduced Klotho expression. We performed rt-PCR on genomic DNA isolated from femurs of 16 weeks-old mice and found that both control and adenine fed *Prx1cre;Klotho*$^{fl/fl}$ mice have undetectable levels of Klotho mRNA expression in the limb bud mesenchyme. No statistically significant changes in Klotho mRNA expression were found between control and adenine fed *Klotho*$^{fl/fl}$ mice, confirming that Klotho inactivation in bone is restricted to the pattern of Prx1-Cre expression.

*Limb bud-specific klotho inactivation prevents CKD induced production of FGF23*

CKD patients and induced-CKD rodent models have high production of FGF23 in osteocytes and markedly elevated circulating FGF23 concentrations (Wesselling-Perry K & Juppner. Bone. 2013. 54(2): 222-9; Shalhoub et al. J Clin Invest. 2012. 122(7):2543-53.). We investigated whether loss of limb specific mKlotho affects FGF23 production in CKD by assessing FGF23 levels in long bones and axial skeleton of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice 8 weeks after CKD induction. As we expected, uremic *Klotho*$^{fl/fl}$ mice had markedly elevated FGF23 transcription in the femur and the lumbar vertebrae when compared to healthy controls. Uremic *Prx1cre;Klotho*$^{fl/fl}$ mice only had increased FGF23 transcription in the lumbar vertebrae where Klotho is still expressed. Femurs of uremic *Prx1cre;Klotho*$^{fl/fl}$ mice showed no changes in FGF23 mRNA expression when compared to control fed *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice. Immunostaining of femurs with FGF23 antibody further showed that uremia induced increases in the expression of FGF23 protein only in the osteocytes of *Klotho*$^{fl/fl}$ mice. As a result, uremic *Klotho*$^{fl/fl}$ mice had significantly more osteocytes that expressed FGF23 protein than all other treatment groups. To investigate whether the resistance of FGF23 transcription in uremic *Prx1cre;Klotho*$^{fl/fl}$ mice is reflected systemically, we measured serum FGF23 levels of mice with the total and intact FGF23 ELISA. Our findings showed that although all uremic mice have elevated serum FGF23 levels, uremic *Prx1cre;Klotho*$^{fl/fl}$ mice have significantly lower concentrations of total and intact serum FGF23 than uremic *Klotho*$^{fl/fl}$ mice. The ratio of intact to total FGF23 in serum was not significantly different between uremic *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice. Moreover, the expression level of Glant3 mRNA in bone was unaffected. These together suggest that conditional deletion of mKlotho does not affect FGF23 degradation. Thus our data provide support for the hypothesis that, in the absence of mKlotho, bone cells become resistant to uremia induced increases in FGF23 transcription (Figure 6).

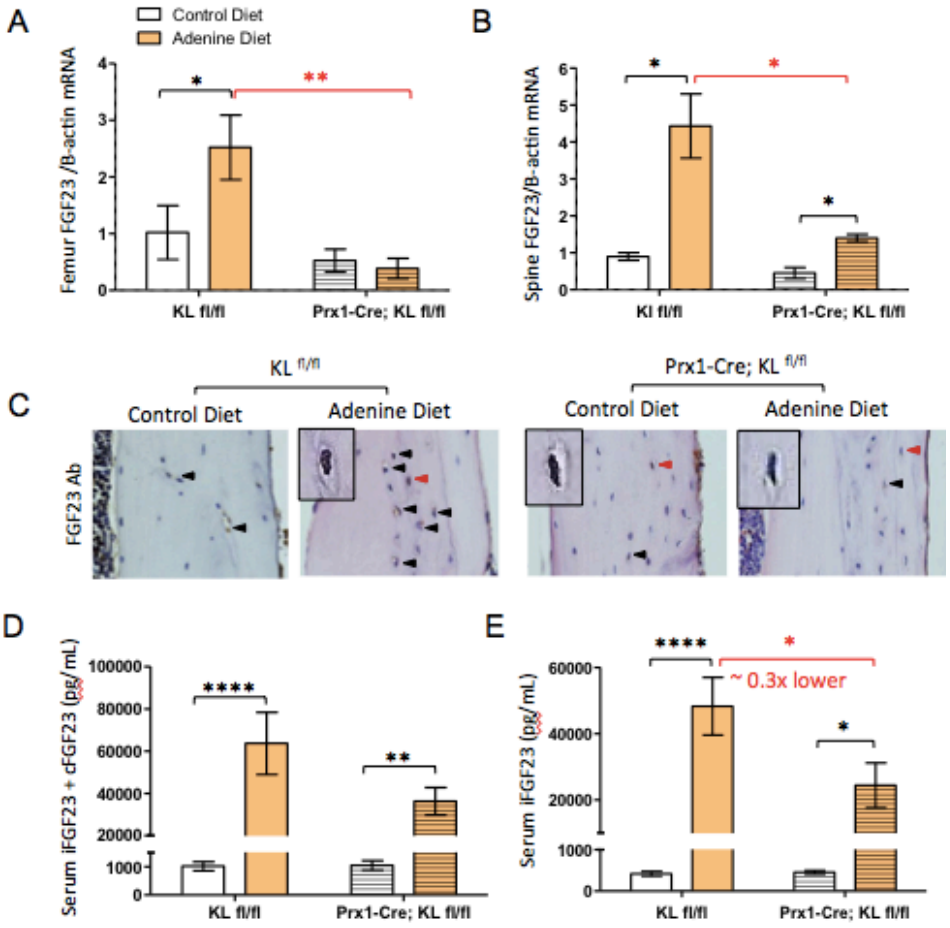



Figure 6: qRT-PCR for Fgf23 in (A) femur and (B) spine of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice fed control diet or adenine diet.
(C) Immunohistochemistry for FGF23 in cortical bone of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice fed control diet or adenine diet at 16 weeks of age. (D) Total and (E) intact FGF23 serum levels of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice fed control diet or adenine diet.

*Uremic Prx1-Cre;Klotho*$^{fl/fl}$ *have lower serum PTH levels & urinary Pi excretion than uremic Klotho*$^{fl/fl}$

FGF23 and PTH are believed to act in concert in CKD so we then investigated whether lower levels of circulating FGF23 in uremic *Prx1cre;Klotho*$^{fl/fl}$ mice affect serum PTH levels. We observed that all adenine fed mice have significantly higher serum PTH levels than control fed mice, with adenine fed *Prx1cre;Klotho*$^{fl/fl}$ mice having significantly lower serum PTH levels than adenine fed *Klotho*$^{fl/fl}$ mice. Interestingly, both serum FGF23 and PTH levels of uremic *Prx1cre;Klotho*$^{fl/fl}$ mice are one third lower than those of uremic *Klotho*$^{fl/fl}$ mice. From published studies we know that both FGF23 and PTH act on the kidney independently to stimulate phosphate wasting by decreasing the expression of the sodium-dependent phosphate co-transporters so we examined NaPi2a and NaPi2c expression. RT-PCR analyses of genomic DNA extracted from kidneys showed that adenine fed mice have suppressed expression of renal genes including Klotho, FGFR1, PTHR1, NaPi2a and NaPi2c, which is consistent with previous reports showing that adenine exposure induces severe renal destruction (Jia et al. BMC Nephrol. 2013. 14:116). In rodents, NaPi2a is the major renal isoform responsible for the reabsorption of approximately 70% of filtered phosphate [18, 19] so we isolated renal BBMV to measure NaPi2a protein expression and could show that adenine fed mice had significantly less NaPi2a protein in the BBMV than control fed mice, and significantly higher urinary phosphate excretion (Figure 7). In comparing across genotypes, adenine fed *Prx1cre; Klotho*$^{fl/fl}$ mice had less pronounced reductions in BBMV NaPi2a protein expression and lower urinary phosphate excretion than uremic *Klotho*$^{fl/fl}$ mice.

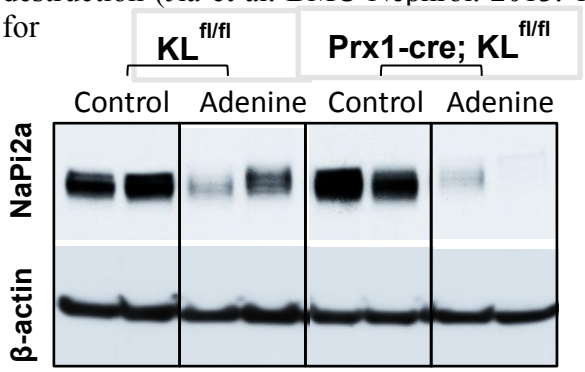



Figure 7: Western blot analysis for Napi2a of renal BBMV

*Uremic Prx1-Cre;Klotho*$^{fl/fl}$ *have higher serum vitamin D and calcium levels than uremic Klotho*$^{fl/fl}$

In the kidney, where Klotho is most abundantly expressed, FGF23 binds to the Klotho-FGFR1 complex to lower $1,25(OH)_2D_3$ levels by inhibiting renal 1α-hydroxylase (Cyp27b1) and stimulating renal 24-hydroxyalase (Cyp24) production. At the same time, PTH increases $1,25(OH)_2D$ levels by stimulating renal 1α-hydroxylase (Cyp27b1) production. As shown in our study, adenine fed *Klotho*$^{fl/fl}$ mice did not show significant changes in

renal 1αOHase or 24OHase expression and had normal serum calcitriol and calcium concentrations. On the contrary, uremic *Prx1cre;Klotho*$^{fl/fl}$ mice had significantly higher renal 1αOHase expression and serum 1,25(OH)$_2$D levels than all other treatment groups with no significant changes in 24OHase expression. Serum 1,25(OH)$_2$D levels increase intestinal calcium absorption and suppress PTH secretion, which is in part why uremic *Prx1cre;Klotho*$^{fl/fl}$ mice were found to be hypercalcemic and have significantly lower serum PTH levels than uremic *Klotho*$^{fl/fl}$ mice (Figure 8).

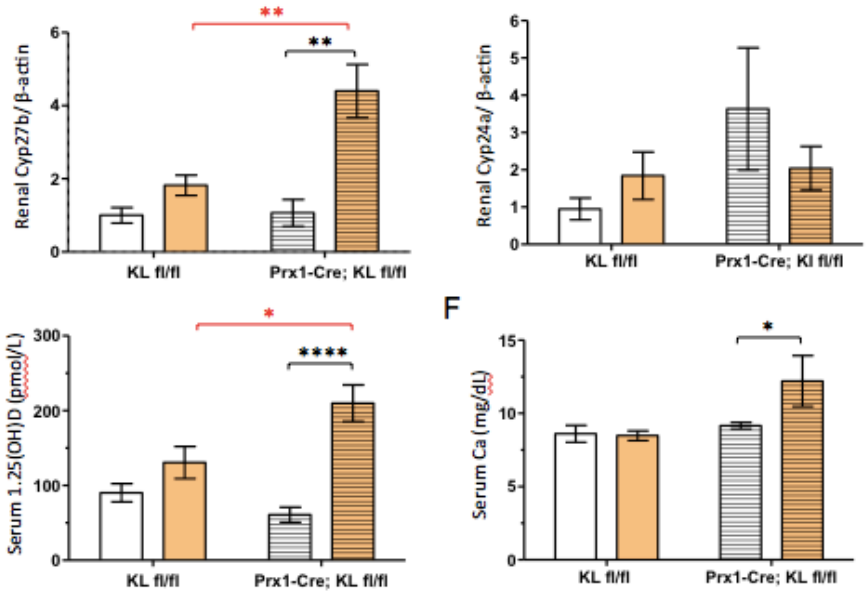



Figure 8: qRT-PCR of renal Cyp27b and Cyp24a of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice fed control diet or adenine diet at 16 weeks of age. Serum 1,25(OH)$_2$D and calcium levels. *p<0.05, **p<0.01, ****p<0.0001

*Findings confirmed in a second model of renal failure*

We validated these results in 5/6 NTX mice. All NTX mice had significantly higher urea concentrations than sham-operated mice, confirming the induction of renal failure. There were no significant differences in serum phosphate levels between groups but, as expected, limbs of 5/6 NTX *Klotho*$^{fl/fl}$ mice had significantly higher FGF23 mRNA expression than those of sham-operated *Klotho*$^{fl/fl}$ mice. Limbs of 5/6 NTX *Prx1cre;Klotho*$^{fl/fl}$ mice, like those treated with adenine, were resistant to uremia induced increases in FGF23 mRNA transcription. As a result, serum iFGF23 levels of *Klotho*$^{fl/fl}$ mice rose from 168.0±8.4 to 902±316.6 after nephrectomy, while those of *Prx1cre;Klotho*$^{fl/fl}$ mice rose modestly from 149 ± 23.9 to 378.2 ± 43.8. All 5/6 NTX mice had significantly higher serum PTH and 1,25(OH)$_2$D levels than sham-operated mice, which is consistent with progressive secondary HPT and altered mineral homeostasis. Serum calcium levels of *Prx1cre;Klotho*$^{fl/fl}$ mice increased significantly after nephrectomy but remained in the normal range, while there were no such increases observed in *Klotho*$^{fl/fl}$ mice (Figure 9).

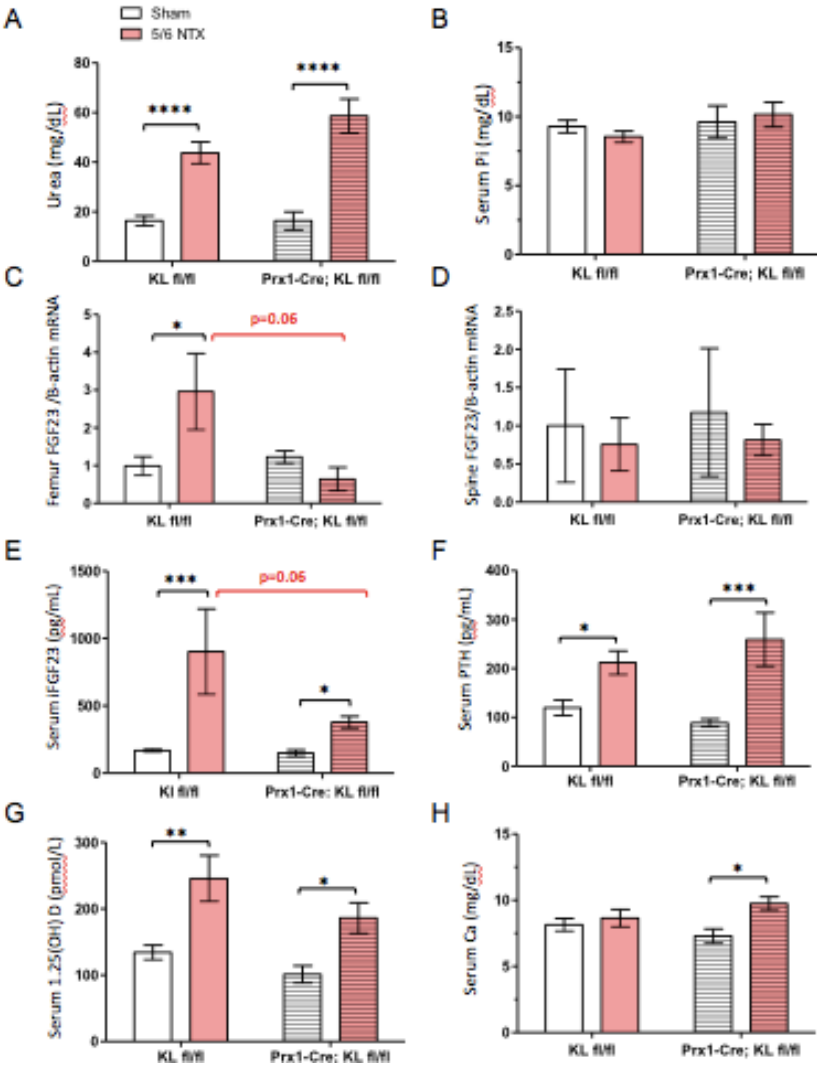



Figure 9: Biochemical analyses of (A) serum urea, (B) serum phosphate, (E) serum iFGF23, (F) serum PTH, (G) serum 1,25(OH)$_2$D, and (H) serum calcium of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice after sham or 5/6 NTX at 16 weeks of age. mRNA expression for Fgf23 in (C) femur and (D) spine of *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice after sham or 5/6 NTX at 16 weeks of age.

*Effects of limb bud-specific Klotho inactiovation on bone*

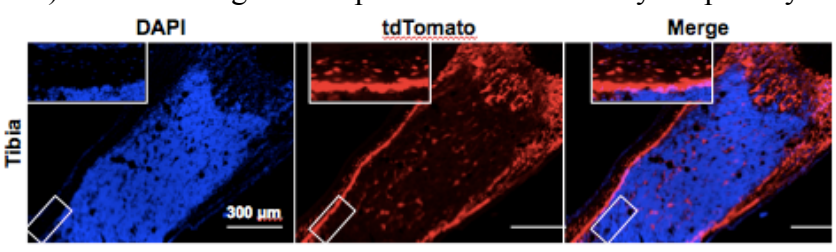
Serum PINP and CTX analyses revealed that all uremic mice have increased bone turnover with no significant differences between uremic *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice. As previously shown (Ferrari et al. BMC nephrology, 2014. **15**:69), adenine fed mice had higher serum PINP and CTX levels than 5/6 NTX mice, suggesting that adenine exposure induces a more severe form of metabolic bone disease. For this reason we used bones of adenine fed mice to characterize how conditional deletion of Klotho affects bone physiology. We show by high-resolution peripheral quantitative computer tomography (pQCT) that adenine fed mice have significantly lower bone mineral density (BMD) and cortical thickness than control fed mice, with greater losses observed in the conditional knockout model. It is well documented that PTH is catabolic in cortical bone and anabolic in trabecular bone, so it is no surprise that adenine fed mice with decreased amount of cortical bone had more trabecular bone than control fed mice. Specifically, the renal osteodystrophy associated with CKD-MBD and secondary hyperparathyroidism manifested in uremic *Klotho*$^{fl/fl}$ mice as significantly higher trabecular bone volume (BV/TV), trabecular thickness (Tb.Th.) and trabecular connectivity (higher Tb.N, lower Tb.Sp) compared with that of healthy controls, while no such effect was observed in uremic *Prx1cre;Klotho*$^{fl/fl}$ mice. As a result, trabecular bone volume, trabecular thickness and trabecular connectivity in distal femurs and lumbar vertebrae of uremic *Prx1cre;Klotho*$^{fl/fl}$ mice were not significantly different from those of healthy controls. Analyses of dynamic histomorphometric parameters revealed that both uremic *Klotho*$^{fl/fl}$ and *Prx1cre;Klotho*$^{fl/fl}$ mice have higher bone formation rate (BFR) and mineral apposition rate (MAR) in the distal femurs and lumbar vertebrae than their respective controls with no significant changes in the amount of mineralized surface. There were no differences in osteoid volume, osteoid thickness, osteoblast surface or osteocyte number between groups at the femur or the lumbar vertebrae. The osteoclast number and surface were significantly higher in the distal femurs, but not lumbar spines, of *Prx1cre;Klotho*$^{fl/fl}$ mice in response to uremia. This would suggest that conditional deletion of Klotho affects osteoclastogenesis.

We are planning to submit this manuscript shortly for publication and hope that this aim will then be completed.

**Aim 3 – *Dmp1cre;Klotho*$^{fl/fl}$ Mice**

Recent experimental data have shown that Klotho is expressed at low levels in osteocytes, but the specific role of Klotho in osteocytes remains unknown. Klotho that is expressed in osteocytes may affect skeletal mineralization and systemic mineral homeostasis. We generated mice with Klotho specifically ablated in osteocytes by crossing *Klotho*$^{fl/fl}$ mice with *DMP1cre* mice to study this possibility. Moreover, emerging data suggest that expressions of Klotho in the kidney and the parathyroid gland are decreased in renal failure and this down-regulation plays an important role in the pathogenesis of CKD-MBD. Thus, we challenged these mutant mice by performing 5/6 nephrectomy in order to determine the potential role of osteocyte expressed Klotho in disease progression.

First, we confirmed Dmp1-Cre expression in bone by crossing *DMP1cre* mice with *tdTomato*$^{fl/fl}$ reporter mice (Figure 10). We also found Dmp1-Cre-mediated expression of tdTomato in the brain, muscle, intestine, and renal collecting duct, but no evidence for the expression of tdTomato in other Klotho-expressing tissues, including the renal proximal and distal tubules and parathyroid gland (data not shown). Consistent with these observations, there were marked reductions (~70%) in Klotho transcripts in the bone (Figure 10) but no changes in expression in the kidney or parathyroid glands.



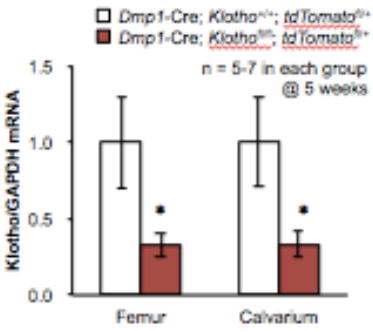




Figure 10: (left) Dmp1-Cre-mediated expression of tdTomato in bone cells.

(right) Efficient deletion of Klotho from femure and calcarium of *Dmp1*-Cre; *Klotho*$^{+/+}$; *tdTomato*$^{fl/+}$ mice

*DMP1cre*;*Klotho*$^{fl/fl}$ mice were indistinguishable from *Klotho*$^{fl/fl}$ littermates and wild-type mice. There was no significant difference in serum levels of calcium, phosphorus, PTH, 1,25-dihydroxyvitamin D, and intact FGF23, as well as urinary excretion of calcium and phosphorus (data not shown). However, *DMP1cre*;*Klotho*$^{fl/fl}$ mice had increased bone volume/total volume (BV/TV), together with increased bone formation and serum levels of bone formation marker PINP at 5 weeks of age (Figure 11).

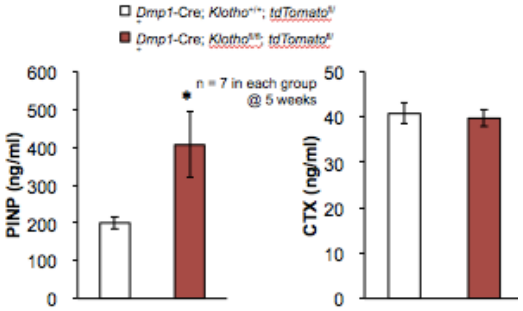



Figure 11: Bone turnover markers at 5weeks of age

RT-PCR analyses of mRNA extracted from the femurs of 5 week old mice revealed that *Prx1cre;Klotho*$^{fl/fl}$ mice have significantly higher expression of osteoblastic and osteocytic markers such as *Col1a1*, *Runx2*, *Osteocalcin*, and *Dmp1* (Figure 12).

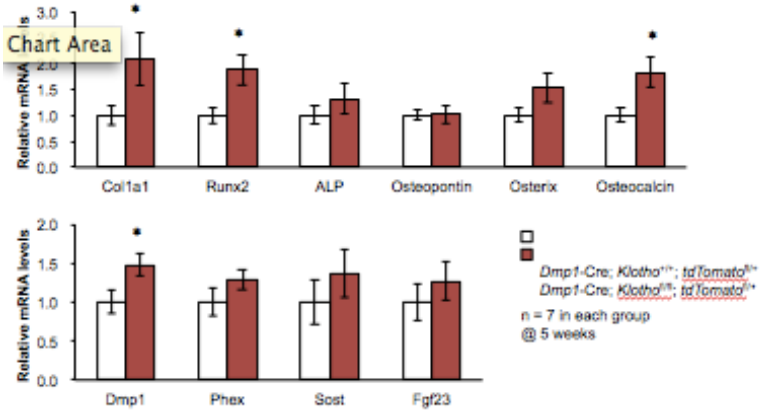



Figure 12: Expression of transcripts related to bone formation and metabolism

We next challenged the mice by inducing renal failure by 5/6 nephrectomy with or without high-phosphate diet. The surgical procedure was performed in 5 week-old mice following a two-step surgical procedure. Briefly, the right kidney was exposed, decapsulated, and two-thirds of the kidney was resected. After a 1-week recovery period, a left total nephrectomy was performed. After two weeks, BUN was quadrupled and serum creatinine was increased by 50% in nephrectomized mice (data not shown), confirming renal failure. For 5/6 nephrectomy plus high-phosphate diet group, high-phosphate diet (containing 1.2% phosphate) was started 1 week after the second surgery. Other groups (sham surgery group and 5/6 nephrectomy group) continued to receive regular diet that contains 0.6% phosphate.

After 5/6 nephrectomy, there were significant increases in serum calcium, PTH, 1,25-dihydroxyvitamin D, and intact FGF23, but there were no significant differences in these values between *DMP1cre*;*Klotho*$^{fl/fl}$ mice and *DMP1cre*;*Klotho*$^{+/+}$ mice (Figure 13). Addition of high-phosphate diet after 5/6 nephrectomy induced a more marked increase in intact FGF23 together with significant increases in serum calcium, PTH, and 1,25-dihydroxyvitamin D, but no significant differences were observed between *DMP1cre*;*Klotho*$^{fl/fl}$ mice and *DMP1cre*;*Klotho*$^{+/+}$ mice (Figure 13).

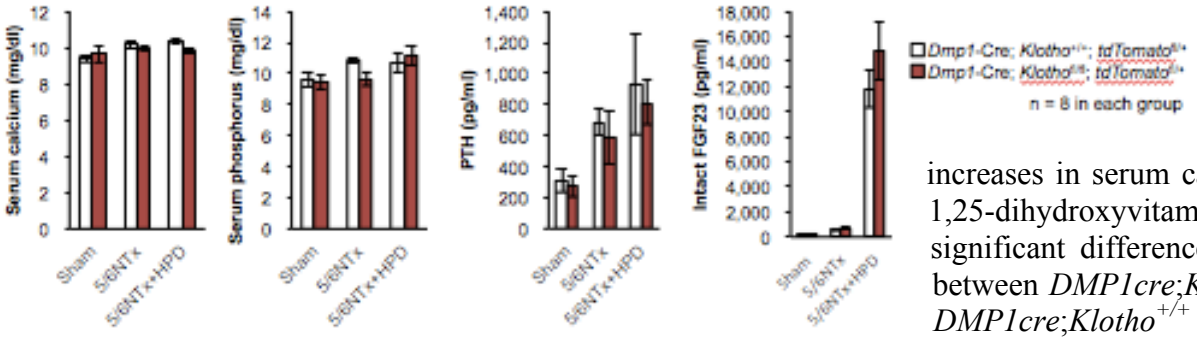



Figure 13: Effect of 5/6NTx w/ or w/o HPD on mineral metabolism

Histomorphometric analyses performed at 12 weeks of age (7 weeks after 2nd surgery) revealed that bone volume/total volume (BV/TV) was increased in *DMP1cre*;*Klotho*$^{fl/fl}$ mice compared to *DMP1cre*;*Klotho*$^{+/+}$ mice after sham surgery (Figure 14), consistent with data on mice at 5 weeks of age. There were no significant increases in serum PINP as well as bone formation marker by RT-PCR analyses at this point (data not shown). The increased bone volume in *DMP1cre*;*Klotho*$^{fl/fl}$ mice remained significant even after 5/6 nephrectomy, but this increase was diminished by 5/6 nephrectomy plus high-phosphate diet (Figure 14).

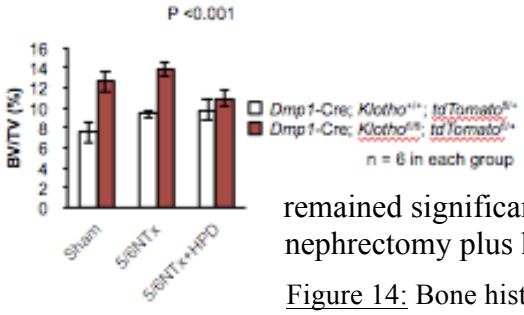



Figure 14: Bone histomorphometry analysis.

We also found that the expression of Klotho in osteocytes was depressed in mice after 5/6 nephrectomy plus high-phosphate diet (Figure 15), as has been shown in the kidney and parathyroid glands from patients with renal failure.

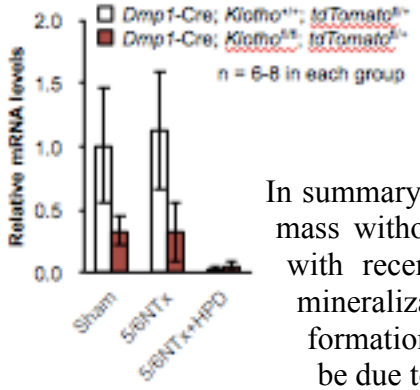



Figure 15: Expression of Klotho in bone cells

In summary, osteocyte-targeted deletion of *Klotho* did increase bone formation and bone mass without significant alterations in mineral metabolism. This finding is consistent with recent data suggesting the effect of Klotho to inhibit vascular and skeletal mineralization in cultured cells. Interestingly, the effect of Klotho deletion on bone formation was diminished after 5/6 nephrectomy plus high-phosphate diet, which may be due to overt renal osteodystrophy and depressed Klotho expression in bone cells.

**Aim 3 – *Col 1( α 1)cre;Klotho*$^{fl/fl}$ vs Runx2cre; *Klotho*$^{fl/fl}$ Mice**

In order to accomplish a more efficient deletion of Klotho in all bone forming cells we have decided to switch from *Col 1( α 1)cre;Klotho*$^{fl/fl}$ to Runx2cre; *Klotho*$^{fl/fl}$-*(Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, Ostermay S, Schinke T, Spanbroek R, Zaiss MM, Angel PE, Lerner UH, David JP, Reichardt HM, Amling M, Schütz G, Tuckermann JP. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor.Cell Metab. 2010 Jun 9;11(6):517-3)*. Runx2 is expressed earlier in the differentiation of bone cells and will therefore increase the effect of Klotho loss by affecting a greater percentage of bone. This will enhance the effects we have already seen in other lines and enable us to delve further into physiology of the phenotype. We have recently obtained our first *Runx2cre;Klotho*$^{fl/fl}$ mutants and are starting to collect tissues and samples for our analyses. We will hopefully complete those studies in this year and will submit the work for publication before the end of the funding.

**Opportunities for Training and Professional Development**

**Dr. Beate Lanske (Principal Investigator)** attended the following conferences

ASBMR CONFERENCE, October 2015

Dr. Lanske, together with Drs. Moe and Hruska, was the Organizer of the ASBMR Pre-Symposium Day *"Crosstalk between Kidney and Bone: From Bench to Bedside*", Seattle, WA. This conference was in conjunction with the ASBMR and was a great success to bring together scientists from the bone and kidney field. More than 400 participants were present.

She is also an active member of the ASBMR Nomination's, Finance, Strategic Planning Committee. She acted as abstract Reviewer for ASBMR, *Paracrine Regulators*.

ASBMR COUNCIL, February and JUNE 2015:

Dr. Lanske attended the winter ASBMR Council meeting in Washington DC and attended the SUMMER council meeting over the phone.

CALCIUM SENSING RECEPTOR SYMPOSIUM, San Diego, March 2015:

Dr. Lanske attended this symposium to learn about the possible interaction of Klotho and the calcium sensing receptor

Importantly, the meetings she attended provided the opportunity to start and continue new and existing collaborations and discuss areas relevant to this grant with colleagues and peers, including FGF23, Klotho, PTH, calcium/phosphate homeostasis and mineralization.

ASN Meeting, San Diego, November 2015:

Dr. Lanske is attending the conference together with Dr. Ide, who will be oresenting her work on deletion of Klotho from the proximal tubules (AIM 2).

Dr. Lanske was asked to evaluate a PhD thesis at McGill University in Montreal, Canada

Since 2014, Dr. Lanske serves also as member on Standing Committee on Promotions, Reappointments, and Appointments at HMS

**Dr. Noriko Ide (postdoctoral fellow) and Dr. Junichi Hanai (collaborator)**

Drs. Ide and Hanai have been working closely together to analyze the phenotype of *Kapcre;Klotho*$^{fl/fl}$ using various techniques. Dr. Ide has learned the techniques for Flow Cytometry analysis and magnetic bead isolation of cells from mice. Dr. Ide has also acquired the surgical technique for creating the (Ischemia-reperfusion induced) acute kidney injury (AKI) mouse model. Dr. Ide attended the ASN conference (Philadelphia) on November 12-16$^{th}$ 2014 and she has been selected to present an oral presentation in the 2015 conference entitled "In Vivo Role of Klotho in the Renal Proximal Tubules". The ASN conference will be in San Diego on November 3$^{rd}$-8$^{th}$, 2015).

Dr. Ide discussed the proximal tubular specific Klotho deletion system with Dr. Lanske and the collaborators Drs. Hannes Olauson and Tobias E. Larsson (Karolinska Institutet, Sweden) on April 24$^{th}$, May 13$^{th}$, June 23$^{rd}$, 2015 via phone conference. Further discussions were held with Dr. Olauson in person on August 6$^{th}$, 2015 at Harvard School of Dental Medicine.

Dr. Ide presented her data within the Lanske Lab prior to ASN:

- ASN conference report (12/3/14)
- Findings and suggestive mechanisms of Klotho on mineral metabolism, which are evidenced by the proximal tubules specific deletion of Klotho (4/16/15)
- In Vivo Role of Klotho in the Renal Proximal Tubules (6/4/15)

**Hao Wang (Research Assistant)**

Mr. Wang has been trained in various techniques, including 5/6 NTX surgery, in order to assist the researchers.

**Katsuhiko Amano (postdoctoral fellow)**
Dr. Amano has been performing all IHC and histological bone analyses for all three aims. He also has been teaching other postdocs in these procedures.

**Hirotaka Komaba (postdoctoral fellow)**
Dr. Komaba has been performing 5/6 nephrectomy and ischemia-reperfusion induced acute kidney injury and has been teaching other postdocs and the assistant in these procedures. He has also developed the techniques for primary cultures of osteocytes derived from mouse long bone._ Dr. Komaba will attend the ASN KIDNEY WEEK 2015 in San Diego to give a lecture on "Effects of medical and surgical parathyroidectomy on bone fragility".

**Dr. Jovana Kaludjerovic (postdoctoral fellow)**
Dr. Kaludjerovic has presented at and/or attended the following conferences:

EUROPEAN CONGRESS OF ENDOCRINOLOGY, May 2015:
Dr. Kaludjerovic was invited to give an oral presentation at this year's European Congress of Endocrinology in the oral communication session titled: Calcium, Vitamin D and Bone.

Dr. Kaludjerovic serves as an ambassador for the European Young Endocrine Scientists Society. In this role she is integrally involved in communicating the opinions, suggestions and expectations of young scientists to European Society of Endocrinology, strategic planning of scientific meetings and recruitment and cultivation of new members. As a result, she organized and chaired a session titled "Sex Drugs and Rocking Hormones" at this year's European Congress of Endocrinology in Dublin, Ireland.

MGH CENTER FOR SKELETAL RESEARCH SYMPOSIUM, June 2015
Dr. Kaludjerovic gave a poster presentation at the Massachusetts General Hospital Center for Skeletal Research Symposium. This provided her a good networking environment with local researchers.

HSDM/HMS/MGH BONE RESEARCH WORKSHOP SERIES
Dr. Kaludjerovic gave an oral presentation titled "Klotho expression is critical for FGF23 production in long bones during renal failure" in the highly prestigious Ether Dome in Boston, MA. Klotho expression is critical for FGF23 production in long bones during renal failure.

She maintains memberships in the American Society for Bone and Mineral Research (ASBMR), American Society for Nutrition (ASN), The Endocrine Society (ENDO), European Society of Endocrinology (ESE), Young Active Research in Endocrinology (YARE), Graduate Women in Science (GWIS), Canadian Association of Postdoctoral Scholars and Harvard Postdoctoral Association.

**Dissemination of Results**

The studies for part of Aim 2 have been submitted and are currently under revision. All of Aim 3 is currently being written up for publication with exception of the work on *Runx2cre;Klotho$^{fl/fl}$* mice.

**Plans for Next Reporting Period**

| | | |
|---|---|---|
| **Aim 1** | ***PTHCre; Klotho$^{fl/fl}$* Mice** | completed |
| **Aim 2** | ***KSPcre; Klotho$^{fl/fl}$* Mice** | eliminated |
| **Aim 2** | ***Six2cre; Klotho$^{fl/fl}$* Mice** | completed |
| **Aim 2** | ***KAPcre;Klotho$^{fl/fl}$* Mice** | submitted for publication |
| **Aim 3** | ***Prx1cre;Klotho$^{fl/fl}$* Mice** | ready for publication |
| **Aim 3** | ***DMP1cre;Klotho$^{fl/fl}$* Mice** | ready for publication |
| **Aim 3** | ***Runx2cre;Klotho$^{fl/fl}$* Mice** | to be started, maybe expanded |

We have recently started to work on this project. The advantage of this new Runx2 cre transgenic mouse line would be that the Klotho deletion would take place in a larger pool of osteoblasts.

## 4. Impact

Nothing to report

## 5. Changes/Problems

**Changes in Approach**

1. We generated *SLC34a1$^{GCE}$cre;Tomato$^{fl/fl}$;Klotho$^{fl/fl}$* and *PEPCKcre;Klotho$^{fl/fl}$* (phosphoenolpyruvate carboxykinase) mice in addition to the *KAPcre;Tomato$^{fl/fl}$;Klotho$^{fl/fl}$* mice for the reasons stated above. All mouse lines were analyzed in parallel and compared to each other. The combined data will be hopefully published shortly.
2. We have started to generate *Runx2cre;Tomato$^{fl/fl}$;Klotho$^{fl/fl}$* mice instead of to the *Col1(α1)*cre mice for reasons stated above.

**Problems**
Nothing to report

**Delays and Planned Resolution**
We anticipate no significant delays.

**Changes Impacting Expenditures**
We anticipate no changes.

**Changes in Use or Care of Subjects, Biohazards, Agents**
There are no changes to report.

## 6. Products

We have an article reporting our results for Aim 3 in review: *In vivo evidence for a role of proximal tubular Klotho in the renal proximal tubules.* Noriko Ide, Hannes Olauson, Tadatoshi Sato, Michael Densmore, Hao Wang, Tobias Larsson, Beate Lanske, *Kidney International.*

Three additional articles are in development for submission before the end of 2015 relating to Aim 3, including two papers regarding *Prx1cre;Klotho$^{fl/fl}$* mice and one regarding *Dmp1cre;Klotho$^{fl/fl}$* mice.

# 7. Participants & Other Collaborating Organizations

## Individuals Working on Project

The following individuals have participated in the project during the reporting period.

| Name | **Beate Lanske, PhD** |
|---|---|
| Project Role | Principal Investigator |
| Researcher Identifier | |
| Nearest Person-Month Worked | 2.4 |
| Contribution to Project | Planning of experiments, reviewing data, writing manuscripts |
| Funding Support | DoD, NIH, HSDM |

| Name | **Junichi Hanai, MD PhD** |
|---|---|
| Project Role | Collaborator |
| Researcher Identifier | |
| Nearest Person-Month Worked | 0.6 |
| Contribution to Project | Technical support and interpretation of data as nephrologist |
| Funding Support | DoD, BIDMC |

| Name | **Noriko Ide, MD PhD** |
|---|---|
| Project Role | Postdoctoral Fellow |
| Researcher Identifier | |
| Nearest Person-Month Worked | 12 |
| Contribution to Project | Aim 2 KapCre-Klotho |
| Funding Support | DoD |

| Name | **Hao Wang** |
|---|---|
| Project Role | Research Assistant |
| Researcher Identifier | |
| Nearest Person-Month Worked | 6 |
| Contribution to Project | Maintenance of mouse lines, genotyping |
| Funding Support | DoD, NIH |

| Name | **Katsuhiko Amano, DDS PhD** |
|---|---|
| Project Role | Postdoctoral Fellow |
| Researcher Identifier | |
| Nearest Person-Month Worked | 5 |
| Contribution to Project | Histological analysis of bones in all aims |
| Funding Support | DoD, NIH |

| Name | **Jovana Kaludjerovic, PhD** |
|---|---|
| Project Role | Postdoctoral Fellow |
| Researcher Identifier | |
| Nearest Person-Month Worked | 6 |
| Contribution to Project | Aim 3 Prx1Cre-Klotho |
| Funding Support | Canadian fellowship |

| | |
|---|---|
| Name | **Hirotaki Komaba, MD PhD** |
| Project Role | Postdoctoral Fellow |
| Researcher Identifier | |
| Nearest Person-Month Worked | 12 |
| Contribution to Project | Aim 3 Dmp1Cre-Klotho |
| Funding Support | Japanese fellowship |

**Changes in Active Support**

Nothing to report.

**Other Organizations**

Nothing to report.

## 8. Special Reporting Requirements

Nothing to report

## 9. Appendices

none